THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 4, Issue of January 22, pp. 2563–2570, 1999

Printed in U.S.A.

Multiple Functional Proteins Are Produced by Cleaving Asn-Gln Bonds of a Single Precursor by Vacuolar Processing Enzyme*

(Received for publication, May 15, 1998, and in revised form, September 10, 1998)

Kenji Yamada‡§, Tomoo Shimada‡§, Maki Kondo‡, Mikio Nishimura‡§, and Ikuko Hara-Nishimura‡¶

From the ‡Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan and the §Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies, Okazaki 444, Japan

Precursor-accumulating vesicles mediate transport of the precursors of seed proteins to protein storage vacuoles in maturing pumpkin seeds. We isolated the precursor-accumulating vesicles and characterized a 100-kDa component (PV100) of the vesicles. Isolated cDNA for PV100 encoded a 97,310-Da protein that was composed of a hydrophobic signal peptide and the following three domains: an 11-kDa Cys-rich domain with four C*XXX*C motifs, a 34-kDa Arg/Glu-rich domain composed of six homologous repeats, and a 50-kDa vicilin-like domain. Both immunocytochemistry and immunoblots with anti-PV100 antibodies showed that <10-kDa proteins and the 50-kDa vicilin-like protein were accumulated in the vacuoles. To identify the mature proteins derived from PV100, soluble proteins of the vacuoles were separated, and their molecular structures were determined. Mass spectrometry and peptide sequencing showed that two Cys-rich peptides, three Arg/Glu-rich peptides, and the vicilin-like protein were produced by cleaving Asn-Gln bonds of PV100 and that all of these proteins had a pyroglutamate at their NH_2 termini. To clarify the cleavage mechanism, *in vitro* processing of PV100 was performed with purified vacuolar processing enzyme (VPE). Taken together, these results suggested that VPE was responsible for cleaving Asn-Gln bonds of a single precursor, PV100, to produce multiple seed proteins. It is likely that the Asn-Gln stretches not only provide cleavage sites for VPE but also produce aminopeptidase-resistant proteins. We also found that the Cys-rich peptide functions as a trypsin inhibitor. Our findings suggested that PV100 is converted into different functional proteins, such as a proteinase inhibitor and a storage protein, in the vacuoles of seed cells.

In higher plants, proprotein precursors of most seed proteins are synthesized on the rough endoplasmic reticulum and are then transported to protein storage vacuoles in maturing seed cells (1–3). We have shown that the vesicles with a density of 1.24 g/cm^3 mediate the delivery of proprotein precursors of seed proteins to the vacuoles (4–6). We have succeeded in isolation of the vesicles from maturing pumpkin seeds and have shown that they contained a large amount of precursors of various seed proteins, including 11S globulin and 2S albumin (7). Thus, these vesicles were designated precursor-accumulating (PAC)[1] vesicles. Recently, we have found that the PAC vesicles contain a type I integral membrane protein with epidermal growth factor-like motifs and have shown that the membrane protein binds to peptides derived from the 2S albumin precursor (8). The membrane protein of the PAC vesicles might function as a sorting receptor for seed protein precursors to the vacuoles.

Just after arriving at the vacuoles, the precursor proteins are converted into their respective mature forms by proteolytic cleavages (6, 9). The posttranslational cleavages occur at the carbonyl sides of Asn residues in precursors of various seed proteins of different plants, including storage proteins, lectins, and toxins, as reviewed by Hara-Nishimura *et al.* (10). We have found an enzyme responsible for maturation of these seed proteins and have designated it vacuolar processing enzyme (VPE) (11, 12). VPE recognizes exposed Asn residues on the molecular surface of the precursor proteins and then cleaves the peptide bonds at the carbonyl sides of the Asn residues (6). The VPE-mediated processing system plays a crucial role in maturation of various seed proteins in protein storage vacuoles.

Our previous study showed that VPE homologs can be separated into two subfamilies: one specific to seeds and the other specific to vegetative organs (13, 14). This is consistent with the fact that the plant vacuoles are classified into two types, protein storage vacuoles in seeds and lytic vacuoles in vegetative organs. A VPE-mediated processing system similar to that in protein storage vacuoles is involved in maturation of vacuolar proteins in lytic vacuoles (15). Vegetative VPE might be responsible for conversion of inactive precursor into their mature active proteins, such as proteinase inhibitors and hydrolytic enzymes (15). Similarly, it is likely that seed VPE also plays a role in the activation of some functional proteins in seeds.

Each of most precursor proteins is composed of a functional domain and an NH_2- and/or COOH-terminal propeptide(s), except for a precursor protein of proteinase inhibitors of tobacco that is processed into five homologous inhibitors and an NH_2-terminal propeptide (16). On the other hand, it is not known whether multiple vacuolar proteins with distinct functions are derived from a single precursor. In this study, we demonstrated that a 100-kDa component of the PAC vesicles (PV100) is converted into multiple proteins with a pyroglutamate at their NH_2 termini by cleaving Asn-Gln bonds of PV100 by the action of VPE, after arrival at protein storage vacuoles. We show here a unique mechanism for vacuolar processing at Asn-Gln cas-

* This work was supported by Grants-in-Aid 10440244, 10163242, and 09267241 for the Research for the Future Program (JSPS-RFTF96L00407) from the Japan Society for the Promotion of Science and for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB019195.

¶ To whom correspondence should be addressed. Tel.: 81-564-55-7500; Fax: 81-564-55-7505; E-mail: ihnishi@nibb.ac.jp.

[1] The abbreviations used are: PAC, precursor-accumulating; PV100, a 100-kDa component of PAC vesicles; VPE, vacuolar processing enzyme; BAPA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl; bp, base pair; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

settes in the precursor sequence to produce aminopeptidase-resistant proteins in the plant vacuoles.

EXPERIMENTAL PROCEDURES

Plant Materials—Pumpkin (*Cucurbita maxima* cv. Kurokawa Amakuri Nankin) seeds were purchased from Aisan Shubyo Seed Co. (Nagoya, Japan). For isolation of PAC vesicles and immunocytochemical analysis, pumpkin seeds were planted in the farm of Nagoya University during the summer season, and cotyledons of the maturing seeds, freshly harvested 22–28 days after pollination, were collected.

Isolation of PAC Vesicles—PAC vesicles were isolated from pumpkin cotyledons at the middle stage of seed maturation essentially as described previously (7). The cotyledons (15 g) were homogenized in a solution (7 ml/g fresh weight of cotyledons) of 20 mM sodium pyrophosphate (pH 7.5), 1 mM EDTA, and 0.3 M mannitol with an ice-chilled mortar and pestle, and the homogenate was filtered through cheesecloth. The filtrate was centrifuged at 3000 × *g* for 15 min and the supernatant was centrifuged again at 8000 × *g* for 20 min at 4 °C. The pellet was suspended in 1 ml of 10 mM Hepes-KOH (pH 7.2), 1 mM EDTA, and 0.3 M mannitol. The suspension was layered on a solution of 28% Percoll (Amersham Pharmacia Biotech) in 10 mM Hepes-KOH (pH 7.2), 1 mM EDTA, and 0.3 M mannitol on a cushion of 2 ml of 90% Percoll. Centrifugation was at 40,000 × *g* for 30 min at 4 °C. The vesicle fraction was centrifuged again in a self-generated Percoll gradient. The resulting vesicle fraction was washed in the above-described Hepes-KOH buffer and used for immunoelectron microscopy and immunoblot analysis.

Isolation of Protein Storage Vacuoles and Purification of Proteins Derived from PV100—Protein storage vacuoles (protein bodies) were isolated from dry pumpkin seeds (50 g) by a nonaqueous isolation method, as described previously (17). Isolated protein storage vacuoles were burst in 100 ml of 10 mM Tris-MES (pH 6.5), 0.1 M sucrose, 1 mM EDTA; sonicated; and then centrifuged at 100,000 × *g* for 1 h at 4 °C to remove insoluble proteins and membranes as a pellet, as described previously (18). Ammonium sulfate was added to the supernatant solution to a concentration of 30% saturation. The mixture was incubated for 1 h at 5 °C and then centrifuged at 200,000 × *g* for 15 min. The ammonium sulfate concentration of the supernatant was then increased to 100% saturation, and the incubation and centrifugation steps were repeated. The precipitate was suspended in 4 ml of a solution of 25 mM sodium acetate (pH 5.5) and 5 mM EDTA and then applied to an Econo-*Pac*I0 DG column (Bio-Rad) to remove ammonium sulfate. The preparation was used as the matrix fraction of the protein storage vacuoles.

The matrix fraction was found to contain a large amount of the 4–6-kDa proteins and the 50-kDa protein that were derived from PV100 by posttranslational cleavage. To purify the 4–6-kDa proteins, the matrix fraction was applied to either a reverse phase column (μRPC C2/C18 PC 3.2/3) on a SMART system (Amersham Pharmacia Biotech) or a reverse phase column (μRPC C2/C18 ST 4.6/100) on an ÄKTA system (Amersham Pharmacia Biotech). Elution was carried out with a gradient starting from 0.065% trifluoroacetic acid in distilled water to 0.05% trifluoroacetic acid in acetonitrile, at a flow rate of 200 μl/min for SMART and at a rate of 500 μl/min for ÄKTA. Each fraction was subjected to immunoblot analysis, mass spectrometry, and digestion by pyroglutamate aminopeptidase followed by automatic Edman degradation, as described below, to determine the molecular structures of these proteins. We also measured trypsin inhibitory activity in the fractions to demonstrate the function of the PV100-derived small proteins.

Determination of NH_2-terminal and Internal Amino Acid Sequences—The PAC vesicles and the matrix fraction of the protein storage vacuoles were subjected to SDS-PAGE, and then the separated proteins were transferred electrophoretically to an Immobilon-P membrane (0.22 mm) (Nihon Millipore Ltd., Tokyo, Japan). After staining of proteins on the blot with Coomassie Blue, the band corresponding to either PV100 or the 50-kDa protein was cut out from the blot and subjected to automatic Edman degradation on a peptide sequencer (model 492, Applied Biosystems Inc.).

To determine the internal sequence of PV100, the separated proteins of the PAC vesicles were stained with Coomassie Blue, and the band corresponding to PV100 on the SDS gels was cut out from the gel, as described previously (19). The gel piece was incubated with 5 μg of V8 protease (Sigma) by the method of Cleveland *et al.* (20). After Tricine-SDS-PAGE (21), the separated peptides were transferred to an Immobilon-P membrane and subjected to automatic Edman degradation.

Digestion with Pyroglutamate Aminopeptidase Followed by Edman Degradation—Each 3 μg of the purified 4–6-kDa proteins of the protein storage vacuoles was digested with 0.3 μg of pyroglutamate aminopeptidase (Boehringer Mannheim) in 20 μl of solution of 0.1 M sodium phosphate (pH 8.0), 5% glycerol, 5 mM dithiothreitol, and 1 mM EDTA for 6 h at 50 °C. The digests were directly subjected to automatic Edman degradation.

Purification of VPE—Protein storage vacuoles (protein bodies) were isolated from castor bean endosperm by a nonaqueous isolation method, as described above. VPE was purified from the soluble fraction of the protein storage vacuoles by using ammonium sulfate precipitation and Con-A Sepharose and MonoS column chromatographies, as described previously (11).

In Vitro Processing by VPE—The PAC vesicles (60 μg of proteins) were incubated with the purified VPE in a solution of 50 mM sodium acetate buffer (pH 5.5) and 50 mM dithiothreitol for 15 h at 37 °C. The reaction was subjected to SDS-PAGE and the separated proteins on the gels were transferred electrophoretically to a polyvinylidene difluoride membrane (0.22 μm) (Nihon Millipore Ltd.). The band corresponding to the 50-kDa protein was cut out from the blot to determine the NH_2-terminal sequence. The membrane piece was incubated with 0.5% polyvinylpyrrolidone for 30 min at 37 °C, followed by digestion by 2.5 μg of pyroglutamate aminopeptidase (Boehringer Mannheim) in 40 μl of solution of 0.1 M sodium phosphate (pH 8.0), 5% glycerol, 5 mM dithiothreitol, and 10 mM EDTA at room temperature for 18 h. After the removal of a pyroglutamate at the NH_2 terminus, the 50-kDa protein on the membrane piece was subjected to automatic Edman degradation on a peptide sequencer (model 492, Applied Biosystems Inc.).

Mass Spectrometry—To determine the exact molecular mass of the 4–6-kDa proteins of the protein storage vacuoles, each fraction that was separated on a SMART system as described above was applied to an API 300 triple quadrupole mass spectrometer (PE SCIEX, Foster City, CA) in positive ion detection mode, equipped with ion spray interface. Samples were dissolved in 0.1% formic acid and 50% acetonitrile and then delivered at 3 μl/min. The sprayer was held at a potential of 4.5 kV. Orifice potential was maintained at 25 V.

Assay of Trypsin Inhibitory Activity—Trypsin inhibitory activity was assayed essentially as described by Cechova (22). α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPA) was used as a substrate of trypsin. One of the PV100-derived small proteins, C2 peptide, purified by a reverse phase chromatography, was dissolved in a solution of 0.1 M Tris-HCl (pH 8.0) and 25 mM $CaCl_2$. After preincubation of the C2 peptide (0–2.4 nmol) with 10 μg of trypsin (Sigma) in a 676 μl of 0.1 M Tris-HCl (pH 8.0) and 25 mM $CaCl_2$ at room temperature for 30 min, 333 μl of a substrate BAPA solution (1 mg/ml) was added to the mixture to start the reaction. After incubation of the mixture at room temperature for 30 min, 100 μl of acetic acid was added to stop the reaction. The residual enzyme activity was measured at 405 nm. The amount of the C2 peptide was estimated from the absorbance at 280 nm, and the molar absorption coefficient at 280 nm of the C2 peptide was computed.

Isolation of cDNA for PV100 and Determination of Nucleotide Sequence—A cDNA library in pBluescript II SK+ (Stratagene, La Jolla, CA) was constructed with the poly(A)$^+$ RNA from maturing pumpkin cotyledons, as described previously (23). Four degenerate primers, 1F (5′-GG(A/C/G/T)GC(A/C/G/T)GG(A/C/G/T)GT(A/C/G/T)GA(C/T)CA-3′), 2F (5′-CA(C/T)GA(C/T)GG(A/C/G/T)TG(C/T)GT(A/C/G/T)-3′), 3R (5′-GG(A/G/T)AT(A/C/G/T)GTCAT(A/C/G/T)AC(A/G)TC-3′), and 4R (5′-TA(G/A)TC(T/C)TT(G/A)AA(T/C)TC(A/C/G/T)CC)-3′), were designed on the basis of the NH_2-terminal and internal amino acid sequences of PV100 and synthesized on a DNA synthesizer (model 394, Applied Biosystems Inc., Foster City, CA). Polymerase chain reaction was performed using a set of the 1F and 4R primers and the cDNA library as a template. A 1454-bp DNA was amplified. A second polymerase chain reaction using a set of 2F and 3R primers and the 1454-bp DNA was performed to amplify a 1340-bp DNA. The 1340-bp DNA was inserted into the T-vector to confirm the nucleotide sequence. The 1340-bp DNA was labeled with [γ-^{32}P]dCTP and Megaprime DNA labeling systems (Amersham Pharmacia Biotech). The cDNA library was screened by colony hybridization using the ^{32}P-labeled DNA as a probe. The isolated cDNA lacked an initiation codon. Subsequently, we amplified DNAs covering the 5′ region of PV100 cDNA using a 5′-Full RACE Core Set (Takara, Tokyo, Japan). Two identical clones were amplified, and the nucleotide sequences were overlapped with the isolated cDNA sequence that lacked an initiation codon.

DNA sequencing was performed with a DNA sequencer (model 377, Applied Biosystems Inc.) and −21M13 forward and M13 reverse fluorescent primers in accordance with the manufacturer's directions. The nucleotide and the deduced amino acid sequences were analyzed with DNA analytical software (Gene Works, IntelliGenetics, Mountain View, CA). The hydrophobicity profile of the amino acid sequences was com-

puted by application of the algorithm of Kyte and Doolittle (24), with a window size of 10 residues. A homology plot was computed with the PAM-250 algorithm (25).

Preparation of Specific Antisera—The isolated PAC vesicles were subjected to SDS-PAGE on a 12.5% polyacrylamide gel with subsequent staining with Coomassie Blue. The band corresponding to the PV100 protein with a molecular mass of 100 kDa was cut out from the gel and gently shaken in phosphate-buffered saline for several hours. The gel was emulsified with complete Freund's adjuvant and injected subcutaneously into a rabbit. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-day intervals. One week after the booster injections, blood was drawn, and the antiserum was prepared.

Immunoblot Analysis—Both the PAC vesicles and protein storage vacuoles were subjected to SDS-PAGE followed by either Coomassie Blue staining or immunoblotting. The purified 4–6-kDa proteins of the protein storage vacuoles were also subjected to immunoblot analysis. The immunoblot was performed essentially as described previously (18). The separated proteins on gels were transferred electrophoretically to a polyvinylidene difluoride membrane (0.22 μm) (Nihon Millipore Ltd., Tokyo, Japan). The membrane blot was incubated overnight with anti-PV100 antibodies that were diluted 2000-fold in a solution of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% (v/v) Tween 20, and 3% (w/v) skim milk. Alkaline phosphatase-conjugated antibodies (Cappel, West Chester, PA) and horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech) that were raised in goat against rabbit IgG were diluted 2000-fold and used as second antibodies.

Immunoelectron Microscopy—Maturing pumpkin seeds were freshly harvested. The cotyledons were vacuum-infiltrated for 1 h with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in 0.05 M cacodylate buffer (pH 7.4). The tissues were then cut into slices of less than 1 mm in thickness with a razor blade and treated for another 2 h with freshly prepared fixative. The isolated PAC vesicles were fixed in 4% paraformaldehyde, 1% glutaraldehyde, 0.3 M mannitol, 1 mM EDTA, and 10 mM Hepes-KOH (pH 7.2) for 1 h at 4 °C. The samples were dehydrated in a graded dimethylformamide series at −20 °C and embedded in LR white resin (London Resin Co. Ltd., Basingstoke, Hampshire, UK). Immunogold labeling procedures were essentially the same as those described previously (26), except for the use of the anti-PV100 antibodies that were diluted 1000-fold in blocking solution (1% bovine serum albumin in phosphate-buffered saline). Protein A-gold (15 nm) (Amersham Pharmacia Biotech) was diluted 40-fold and used. The ultrathin sections were examined with a transmission microscope (model 1200EX) (JEOL, Tokyo, Japan) at 80 kV.

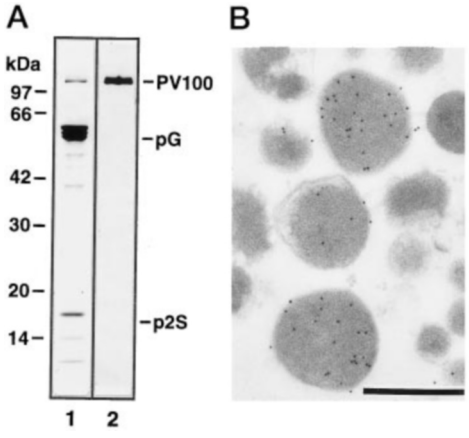


FIG. 1. **PV100 is one of the major proteins in the PAC vesicles from maturing pumpkin seeds.** *A,* isolated PAC vesicles were subjected to SDS-PAGE and subsequent staining with Coomassie Blue (*lane 1*) or immunoblot with anti-PV100 antibodies (*lane 2*). *pG* and *p2S* represent proprotein precursors of 11S globulin and 2S albumin, respectively. The molecular mass of each marker protein is given on the *left* in kDa. PAC vesicles were isolated from the cotyledons at the middle stage of seed maturation of pumpkin. *B,* immunogold labeling of the isolated PAC vesicles with anti-PV100 antibodies. *Bar,* 500 nm.

RESULTS

PV100 Is a 100-kDa Protein Component of PAC Vesicles—We have shown that the PAC vesicles are responsible for the intracellular transport of precursors of major seed proteins, including 11S globulin and 2S albumin, to protein storage vacuoles in maturing pumpkin seeds (4, 6–8). The PAC vesicles were highly purified from cotyledons of maturing pumpkin seeds. Electron microscopy revealed that each PAC vesicle contained an electron-dense core with a diameter of 300–500 nm and that the isolated vesicles were barely contaminated by other cellular components (Fig. 1*B*). Fig. 1*A* (*lane 1*) shows the protein components of the vesicles that were separated on an SDS-gel with Coomassie Blue staining. Three major proteins were found in the PAC vesicle fraction. Two of them have been shown to correspond to proglobulin, a proprotein precursor of 11S globulin (4), and to pro2S albumin, a proprotein precursor of 2S albumin (6), as indicated by *pG* and *p2S* in Fig. 1*A* (*lane 1*), respectively. The third component of the PAC vesicles, with

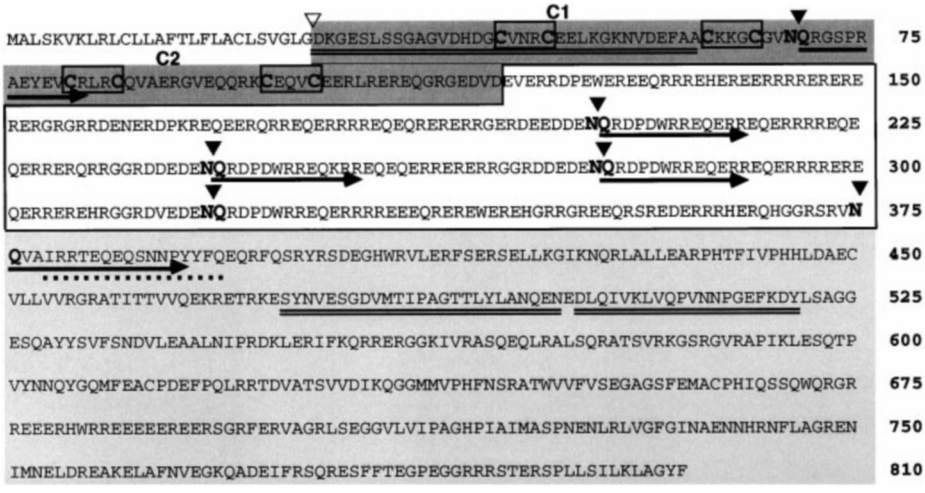


FIG. 2. **Deduced amino acid sequence from a cDNA that encodes PV100.** Isolated cDNA encodes a 97,310-Da protein of 810 amino acids, which consists of a hydrophobic signal peptide followed by the PV100 sequence. The NH_2-terminal sequence and two internal sequences of PV100 that were determined are indicated by *double underlining*. An *open triangle* indicates a cleavage site of a signal peptide. The PV100 sequence was divided into three domains: an 11-kDa Cys-rich domain (indicated by *dark shading*) with four C*XXX*C motifs (enclosed in the *small boxes*), a 34-kDa Arg/Glu-rich domain (enclosed in the *large box*), and a 50-kDa vicilin-like domain (indicated by *light shading*). The *arrows* indicate the determined NH_2-terminal sequences of PV100-derived mature proteins that had been digested by pyroglutamate aminopeptidase (see Figs. 6 and 8), and a *dotted line* indicates the NH_2-terminal sequence of the vicilin-like protein from dry seeds. *Boldfaced NQ* (Asn-Gln) stretches, marked with a *closed triangle,* represent posttranslational processing sites to produce multiple seed proteins, each with a pyroglutamate at its NH_2 terminus. The nucleotide sequence has been submitted to the DNA Data Bank of Japan and GenBank™ with the accession number AB019195.

a molecular mass of 100 kDa, was designated PV100.

For immunochemical characterization of PV100, polyclonal antibodies were raised against the PV100 protein. An immunoblot of the PAC vesicles with the anti-PV100 antibodies showed that the antibodies specifically recognized PV100 on the blot, as shown in Fig. 1*A* (*lane 2*). Immunoelectron microscopy of the isolated PAC vesicles with the anti-PV100 antibodies shows that gold particles are inside the PAC vesicles (Fig. 1*B*). These results indicate that PV100 is localized in the PAC vesicles together with proproteins of the major seed proteins, 11S globulin and 2S albumin.

PV100 Is Composed of Three Domains—The next issue to be solved was the molecular structure of PV100 to clarify the manner of posttranslational cleavage. We determined the amino acid sequences of the NH_2 terminus and two internal fragments of PV100 and then isolated a cDNA with a 4.3-kb insert from the library of maturing pumpkin seeds based on the amino acid sequences. The cDNA encoded a 97,310-Da protein of 810 amino acids, as shown in Fig. 2. The determined NH_2-terminal amino acid sequence, DKGESLSSGAGVDHDGCVNRCEELKG*X*NVDEFAA (*X*, not determined), and the two internal sequences, *X*YNVESGDVMTIPAGTTLYLANQEN and DLQIVKLVQPVNNPGEFKDY, were found in the deduced sequence (Fig. 2, *double-underlined* sequences), indicating that this clone was a cDNA for PV100. The deduced primary structure of the PV100 protein was composed of a hydrophobic signal peptide followed by the PV100 sequence. The NH_2-terminal sequence of PV100 revealed that the signal peptide is cleaved off co-translationally on the carbonyl side of Gly^{27}, as indicated in Fig. 2 (*open triangle*). The cleavage site was consistent with that predicted by application of the rules of Von Heijne (27).

The PV100 sequence was composed of three domains: an 11-kDa Cys-rich domain, a 34-kDa Arg/Glu-rich domain, and a 50-kDa vicilin-like domain, as shown in Fig. 2. The Cys-rich domain contained four C*XXX*C motifs (Fig. 2, *boxes*) of two Cys residues separated by three other amino acids. The 50-kDa domain (Fig. 2, *light shading*) exhibited a 30–35% identity in amino acids to the vicilin homologs, pea vicilin (28), soybean β-conglycinin (29), and jack bean canavalin (30). Precursors of vicilin homologs of pea, soybean, and jack bean are composed of a signal peptide followed by a vicilin domain, whereas the precursors of cacao vicilin (31) and upland cotton α-globulin-A (32) have six and four C*XXX*C motifs, respectively, preceding a vicilin domain. However, the amino acid sequences around the C*XXX*C motifs of pumpkin, cacao, and cotton exhibited a very low similarity to each other.

The Arg/Glu-rich domain of PV100 is composed of 37 mol % Arg and 27 mol % Glu, as shown in Fig. 2 (*large box*). It should be noted that this domain is unique to pumpkin PV100. A homology plot of PV100 shows that six homologous repeats are found in the Arg/Glu-rich domain, as shown in Fig. 3*A*. An alignment revealed that the six homologous repeats were separated by Asn-Gln (Glu) sequences (Fig. 3*B*). The six repeats rich in Arg and Glu were designated RE1–RE6 in order from the NH_2 terminus. They show a sequence homology to pumpkin basic peptide of 36 amino acids that was isolated from pumpkin (*C. maxima* cv. Mexican-papitas) seeds (33). In particular, the 36-amino acid form of RE3 that was found in the seeds could be identical to the pumpkin basic peptide (Fig. 3*C*, discussed below).

Multiple Seed Proteins That Are Derived from PV100 Are Accumulated in Protein Storage Vacuoles—Our previous works have shown that both proglobulin and pro2S albumin are transported from the PAC vesicles to the protein storage vacuoles and then are converted into their respective mature forms (4, 6). This raises the question of whether PV100 is also incorporated into the protein storage vacuoles. Immunocytochemistry of the maturing pumpkin seeds with the anti-PV100 antibodies answered this question. Electron-dense PAC vesicles with diameters of 300–500 nm and protein storage vacuoles composed of crystalloids of 11S globulin and the matrix were observed in the cells, as shown in Fig. 4*A*. Gold particles can be seen distributed in the vacuolar matrix region, the PAC vesicles, and the rough endoplasmic reticulum. In contrast, none of the vacuolar crystalloid, the lipid bodies, the mitochondria, or the cell wall was labeled with gold particles. These results suggested that PV100, which is synthesized on rough endoplasmic reticulum, is transported to PAC vesicles and then to protein storage vacuoles.

The molecular structure of PV100 implies that multiple seed proteins are derived from PV100. The next question is whether PV100 is processed to make such seed proteins in the protein

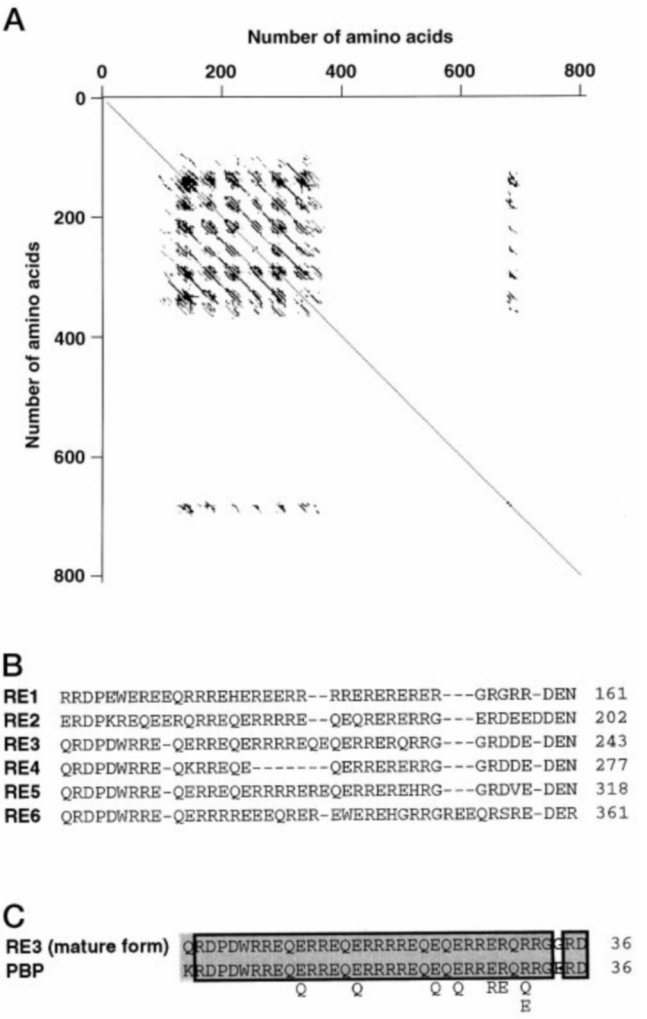


FIG. 3. **Homology plot of PV100 and an amino acid alignment of the six homologous repeats in the Arg/Glu-rich domain.** *A,* a homology plot was performed with the PAM-250 algorithm (25) with a window of 10 residues. Each pair of windows that exhibited more than 35% identity in amino acids is indicated by a *dot* in the matrix. Six homologous repeats were found in the Arg/Glu-rich domain. *B,* Asn-Gln/Glu bonds separate the Arg/Glu-rich domain into six repeats (see Fig. 9). The six Arg/Glu-rich repeats that were designated RE1 to RE6 in order from the NH_2 terminus were aligned. Numbers on the *right* side of each sequence refer to the positions of the amino acids starting from the initiation Met. *C,* the mature RE3 peptide (see Fig. 6*B*) was aligned with the sequence of pumpkin basic peptide, where the second and the third possible amino acids are also shown, as reported by Naisbitt *et al.* (33). Both peptides are composed of 36 amino acids, as indicated on the *right* side of each sequence. *Boxes* enclose identical amino acids, and *shading* indicates homologous amino acids.

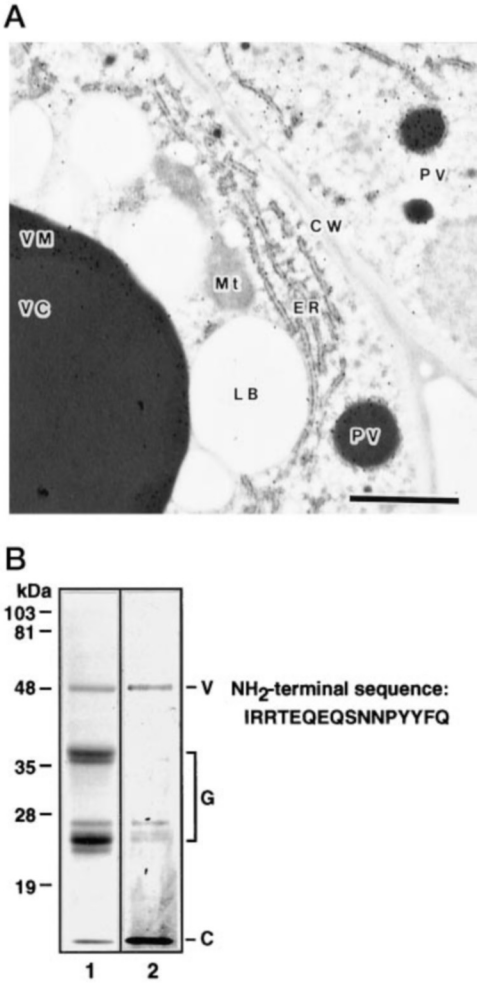


FIG. 4. **PV100-derived proteins are localized in protein storage vacuoles in pumpkin seeds.** *A,* immunoelectron micrograph of maturating pumpkin seeds after staining with anti-PV100 antibodies. Gold particles were distributed in the PAC vesicles (*PV*), the matrix region (*VM*) of protein storage vacuoles and ER. *VC,* vacuolar crystalloid composed of 11S globulin; *Mt,* mitochondrion; *LB,* lipid body; *CW,* cell wall. *Bar,* 1 μm. *B,* isolated protein storage vacuoles (protein bodies) from dry pumpkin seeds were subjected to SDS-PAGE and subsequent staining with Coomassie Blue (*lane 1*) or immunoblot with anti-PV100 antibodies (*lane 2*). PV100-derived proteins, the 50-kDa vicilin-like protein (*V*) and a ~6-kDa C2 peptide (*C*) were detected on the blot. The determined NH_2-terminal sequence of the 50-kDa vicilin-like protein is shown. *G* represents the 11S globulin. The molecular mass of each marker protein is given on the *left* in kDa.

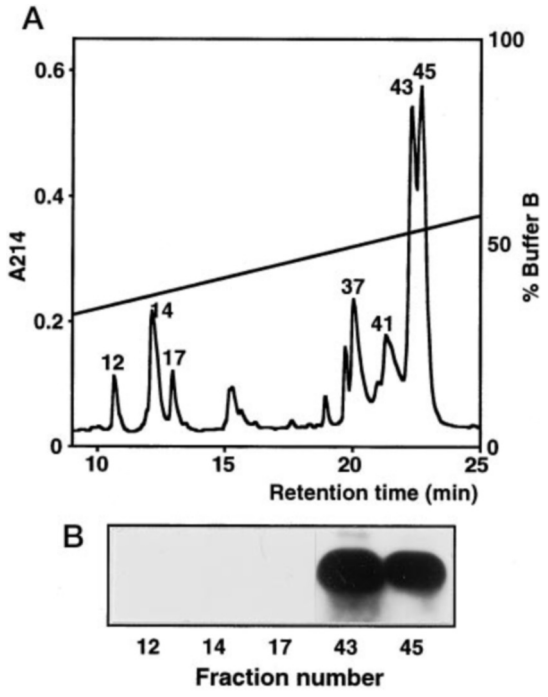


FIG. 5. **An HPLC profile of PV100-derived peptides from the protein storage vacuoles.** *A,* soluble fraction of the protein storage vacuoles that contained the PV100-derived peptides was applied to a reverse phase column. Elution was carried out with a gradient starting from 0.065% trifluoroacetic acid in distilled water to 0.05% trifluoroacetic acid in acetonitrile. Chromatography was monitored in terms of absorbance at 214 nm. *B,* immunoblot analysis of each peak fraction with anti-PV100 antibodies.

storage vacuole. To answer this question, the protein storage vacuoles were isolated from dry pumpkin seeds and subjected to SDS-PAGE followed by immunoblot analysis, as shown in Fig. 4*B*. The vacuoles contained a large amount of 11S globulin (Fig. 4*B, lane 1*). On the immunoblot with anti-PV100 antibodies, two bands, corresponding to a 50-kDa protein and a <10-kDa small protein(s), were detected (Fig. 4*B, lane 2*). The NH_2-terminal sequence of the 50-kDa protein was IRRTEQEQSNNPYYFQ, which corresponds to a sequence starting from the fourth amino acid of the vicilin-like domain, as indicated in Fig. 2, *dotted line* (discussed below). These results suggested that the 50-kDa vicilin-like protein and <10-kDa small protein(s) were produced from PV100 and were accumulated in the protein storage vacuoles.

To identify the <10-kDa small protein(s), soluble proteins of the protein storage vacuoles were separated by HPLC, as shown in Fig. 5*A*. Each peak fraction of the HPLC was subjected to both mass spectrometry and automatic Edman degradation (Fig. 6). Fractions 37 and 41 contained the known *C. maxima* trypsin inhibitor (34), which is not related to PV100. The NH_2 termini of all proteins of fractions 12, 14, 17, 43, and 45 were blocked. When digested by pyroglutamate aminopeptidase, each protein gave an NH_2-terminal amino acid sequence that was consistent with the sequence starting from the second residues of the respective small protein derived from PV100, as indicated in Figs. 2 and 6*B* (*arrow*). Fractions 12, 14, and 17 corresponded to RE4, RE3, and RE5 of the Arg/Glu-rich domain, respectively, and fractions 43 and 45 corresponded to the latter half (C2) of the Cys-rich domain (Fig. 6). Interestingly, all of these small proteins had a pyroglutamate at their NH_2 termini. It should be noted that an Asn residue always preceded all Gln residues to be converted into a pyroglutamate, as indicated in Fig. 2 (*boldfaced NQ* in the PV100 sequence) (discussed below).

The observed molecular masses of fractions 12, 14, and 17 showed good agreement with the theoretical masses of sequence d of RE4, sequence c of RE3, and sequence e of RE5, respectively, each of which has a pyroglutamate at the NH_2 terminus and an Asp residue at the COOH terminus (Fig. 6). Thus, two steps of processing might be required to produce the mature forms of RE peptides from PV100: the first is cleavage at Asn-Gln bonds of PV100, and the second is trimming 2 or 5 amino acids off at the COOH termini of RE intermediates. All of the mature peptides of the Arg/Glu-rich RE3, RE4, and RE5 found in seeds are basic ones with estimated pIs of 11.90, 11.54, and 10.20, respectively. This is in contrast to the neutral pIs of RE intermediates before trimming their COOH-terminal few amino acids.

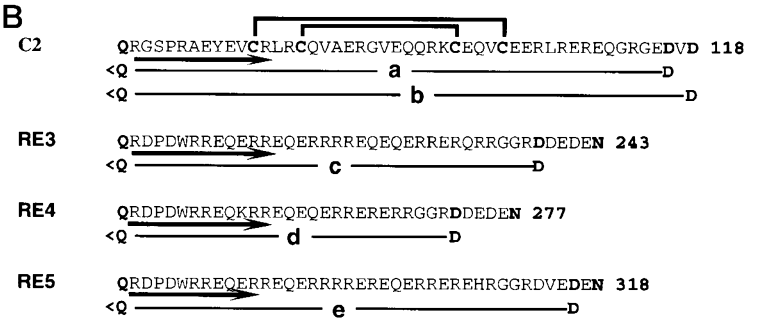
The observed molecular masses of fractions 43 and 45 also showed good agreement with the theoretical masses of sequences a and b of C2 from the Cys-rich domain, respectively, indicating that each sequence has a pyroglutamate at the NH_2 terminus and two intramolecular disulfide bonds (Fig. 6). The

FIG. 6. **Molecular structures of PV100-derived peptides from the protein storage vacuoles.** *A,* molecular masses of the peptides in each HPLC fraction, as shown in Fig. 5, were determined by mass spectrometry. Theoretical molecular masses of RE3, RE4, and RE5 that had a pyroglutamate (<Q) at their NH_2 termini are consistent with the observed values. The number of disulfide bonds in the C2 peptide was determined to be 2. *B,* primary structures of peptide components of fractions 12, 14, 17, 43, and 45 were determined to be *sequence d* (RE4), *sequence c* (RE3), *sequence e* (RE5), and *sequences a* and *b* (C2), respectively. The determined NH_2-terminal sequences after digestion by pyroglutamate aminopeptidase are indicated by *arrows* below the respective sequences. Numbers on the *right* side of each sequence refer to the positions of the amino acids starting from the initiation Met of PV100. The disulfide bridges were deduced from the data of buckwheat trypsin inhibitor that exhibits a characteristic similar to the C2 peptide and has two C*XXX*C motifs and two disulfide bridges (35).

A

Fraction number	Observed mass	Name of peptide	Sequence in (B)	NH_2 terminus	Number of disulfide bonds	Theoretical mass
12	3876.52±0.71	**RE4**	d	**<Q**	0	3877.00
14	4888.00±0.50	**RE3**	c	**<Q**	0	4888.03
17	5242.17±0.90	**RE5**	e	**<Q**	0	5241.34
43	5615.54±0.70	**C2**	a	**<Q**	2	5615.02
45	5829.90±0.80	**C2**	b	**<Q**	2	5829.23

B



disulfide bridges are deduced from the data of buckwheat trypsin inhibitor, an allergenic protein, that exhibits a similar characteristic to the C2 peptide and has two C*XXX*C motifs and two disulfide bridges (35).

To explore function of the C2 peptide, we examined trypsin inhibitory activity of the C2 peptide using BAPA as a substrate of trypsin. The C2 peptide was highly purified. Mass spectrometry showed that the final preparation of the C2 peptide used for the assay was not contaminated by *C. maxima* trypsin inhibitor. We found that the C2 peptide had an inhibitory activity against trypsin, as shown in Fig. 7. Ten μg of trypsin was completely inhibited by 1.2 nmol of C2 peptide. The C2 peptide of 49 amino acids exhibits an 18% identity in amino acids to buckwheat trypsin inhibitor of 51 amino acids. In contrast to the low identity between the two sequences, they have a similar characteristic in the presence of two C*XXX*C motifs in their sequences. The reactive site of buckwheat trypsin inhibitor for trypsin was reported to be Arg^{19}, between the two C*XXX*C motifs (35). The C2 peptide conserves Arg^{21}, between the two C*XXX*C motifs (Fig. 6*B*), and the residue might be the reactive site for trypsin (discussed below).

None of RE1, RE2, RE6, or the former half (C1) of the Cys-rich domain was detected in protein storage vacuoles. They might be degraded in the vacuoles during seed maturation (discussed below). These results indicated that ~6-kDa C2, ~5-kDa RE3, ~4-kDa RE4, and ~5-kDa RE5 are accumulated in the protein storage vacuoles. Fig. 5*B* shows an immunoblot of RE3 (fraction 14), RE4 (fraction 12), RE5 (fraction 17), and C2 (fractions 43 and 45) with anti-PV100 antibodies. Surprisingly, the polyclonal antibodies recognized C2 peptide efficiently, but no RE peptides at all appeared on the blot. These results suggested that the antigenicity of the Cys-rich peptides was much higher than that of the extremely hydrophilic Arg/Glu-rich peptides. On the immunoblot of protein storage vacuoles, the signal corresponding to <10 kDa might be caused by C2 peptide (Fig. 4*B*). The intensity of the signal was much higher than that of the 50-kDa vicilin-like protein. It seems likely that such C*XXX*C motifs cause allergy to animals as buckwheat trypsin inhibitor does (35).

VPE Mediates the Conversion of PV100 into Multiple Seed Proteins—We previously showed that VPE is involved in maturation of various seed proteins in the protein storage vacuoles by cleaving a peptide bond on the carbonyl side of Asn residues

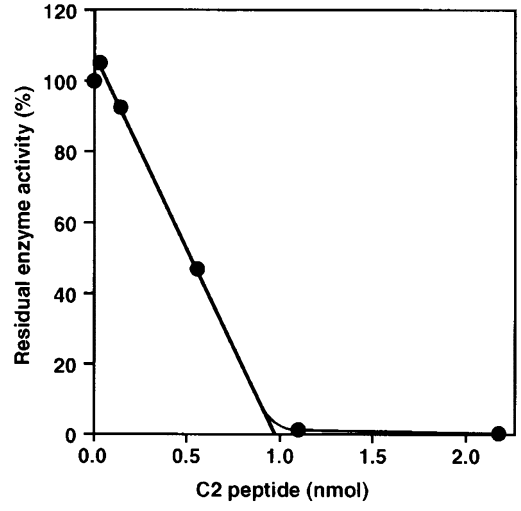


FIG. 7. **PV100-derived C2 peptide functions as a trypsin inhibitor.** The C2 peptide was highly purified from soluble fraction of the protein storage vacuoles of pumpkin seeds by HPLC. The reaction mixture contained 0–2.4 nmol of the C2 peptide, 10 μg of trypsin and 333 μg of BAPA in a 0.9-ml solution of 0.1 M Tris-HCl (pH 8.0) and 25 mM $CaCl_2$ (see under "Experimental Procedures"). The residual enzyme activity was monitored with absorbance at 405 nm.

(6, 10, 15). This raised the question of whether VPE mediates the proteolytic processing of PV100. To answer this question, we performed an *in vitro* processing of PV100 by the purified VPE from castor bean seeds. We used proproteins in the isolated PAC vesicles as substrates, including PV100, proglobulin, and pro2S albumin, as shown in Fig. 1*A* (*lane 1*). After incubation of these proteins with the purified VPE, the amount of PV100 decreased in association with the increase of the amount of a 50-kDa protein and <10-kDa proteins, as shown in Fig. 8. The <10-kDa proteins contained not only PV100-derived small proteins but also 2S albumin, composed of 3.8- and 8.0-kDa subunits, which was produced from pro2S albumin. This indicated that VPE was involved in the conversion of PV100 into the 50-kDa protein and the <10-kDa proteins.

The 50-kDa protein was subjected to automatic Edman degradation after digestion by pyroglutamate aminopeptidase (Fig. 8). The determined NH_2-terminal sequence, <QVAIR-

RTEQEQSNNPY, was found in the sequence of PV100, as in Fig. 2 (*arrow*). The NH_2-terminal sequence determined after *in vitro* processing was consistent with that of the 50-kDa protein accumulated in maturing seeds (data not shown). This suggests that processing similar to the *in vitro* processing of PV100 occurs by endogenous VPE during seed maturation. The result indicated that VPE mediated the production of the 50-kDa vicilin-like protein by cleaving an Asn^{375}-Gln^{376} bond of PV100. The cleavage was consistent with the substrate specificity of VPE toward Asn residues. Further degradation to remove NH_2-terminal three amino acids must occur at the later stage of seed maturation and produce a mature 50-kDa vicilin-like protein with the NH_2-terminal sequence, IRRTEQEQSNNPYYFQ (Figs. 2 and 4*B*). We previously reported that protein storage vacuoles accumulated not only VPE but also aspartic proteinase (36). It seems likely that such aspartic proteinase might be involved in the proteolytic trimming.

It should be noted that most processing occurs at Asn-Gln bonds in the hydrophilic region of PV100, and all of the mature proteins, the 50-kDa protein, and C2 and RE peptides have a pyroglutamate at their NH_2 termini, as shown in Fig. 9. Similar VPE-mediated processing of PV100 might occur to produce the multiple seed proteins (discussed below).

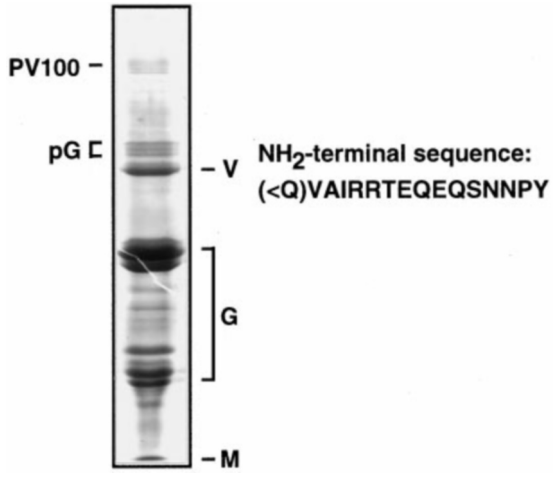


FIG. 8. ***In vitro* processing of PV100 by purified VPE produced the vicilin-like protein.** PAC vesicles that contained PV100 were incubated with the purified VPE and then subjected to SDS-PAGE followed by staining with Coomassie Blue. The resultant band corresponding to 50-kDa vicilin-like protein (*V*) that had been blotted to a polyvinylidene difluoride membrane was incubated with pyroglutamate aminopeptidase and then was subjected to automatic Edman degradation. The determined NH_2-terminal sequence corresponds to the sequence in PV100, as indicated by an *arrow* in Fig. 2. *pG* and *G* represent proglobulin and 11S globulin, respectively. The <10-kDa band (*M*) contained a mixture of the PV100-derived peptides and 2S albumin subunits that had been produced from pro2S albumin by VPE in the reaction.

DISCUSSION

PV100 Is a Unique Precursor to Multiple Functional Proteins—The present study demonstrates that PV100 is not only a precursor of vicilin storage protein but also a precursor of the Arg/Glu-rich RE peptides and a precursor of the Cys-rich C2 peptide that acts as a trypsin inhibitor. PV100 is a unique precursor for multiple seed proteins with different functions.

The C2 peptide was shown to have trypsin inhibitory activity. However, the sequence of the C2 peptide has no homology to known trypsin inhibitors, including members of squash trypsin inhibitor family (34), except for buckwheat trypsin inhibitor, showing a 18% identity to the C2 peptide. Interestingly, despite such low homology in primary structure, the higher structure of the C2 peptide might be analogous to that of buckwheat inhibitor. It has been shown that the buckwheat trypsin inhibitor forms a hairpin structure, in which two C*XXX*C motifs are linked by two disulfide bonds, and that Arg^{19}, between the two C*XXX*C motifs, is the reactive site for trypsin (35). Similarly, all four Cys residues of the C2 peptide formed two disulfide bonds, and Arg^{21} is found between the two C*XXX*C motifs (Fig. 6*B*). The result suggests that both the C2 peptide and buckwheat inhibitor belong to a novel family of trypsin inhibitors. These inhibitors might play a role in protecting the seeds from animals.

Among the Arg/Glu-rich RE peptides, the mature RE3 with the highest pI value (pI 11.90) shows the highest content in pumpkin seeds. We compared the RE3 composed of 36 amino acids with the pumpkin basic peptide that has been shown to be toxic to mouse B-16 cells (33) (Fig. 3*C*). The cytotoxic basic peptide was composed of 36 amino acids, and the probable amino acid sequence of the peptide was reported by Naisbitt *et al.* (33). Both sequences are identical to each other, except for two residues. It is likely that the mature RE3 accumulated in the vacuoles of pumpkin seeds might be identical to the cytotoxic basic peptide that was characterized by Naisbitt *et al.* (33). This suggests that the mature RE3 might function as a toxin to prevent animals from eating the seeds.

Most vacuolar proteins are synthesized as a proprotein precursor on the rough endoplasmic reticulum and are then transported to vacuoles. The vacuolar targeting signals have been shown to be present in the propeptides of some vacuolar proteins, including barley aleurain (37), barley lectin (38), sweet potato sporamin (39), and tobacco chitinase (40). It has been thought that the propeptides are cleaved off and degraded after

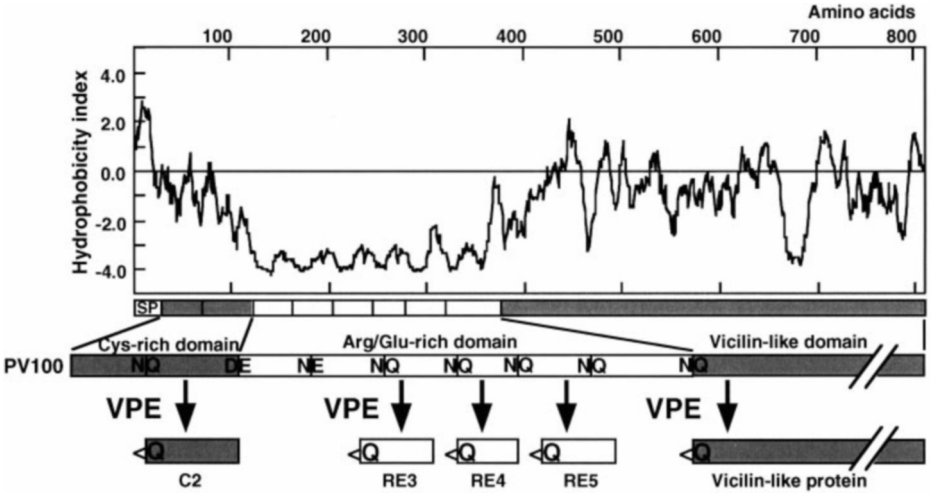


FIG. 9. **Hydropathy profile of PV100 and a hypothetical mechanism for the VPE-mediated cleavage at Asn-Gln bonds to produce multiple seed proteins.** The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle (24) with a window of 10 residues. VPE is responsible for maturation of multiple seed proteins by cleaving Asn-Gln bonds that are found in the hydrophilic region of the PV100. Gln at the new NH_2 termini of the mature proteins might be spontaneously converted into pyroglutamate (<*Q*) under the acidic condition in the vacuoles. The cysteine-rich C2 peptide, the Arg/Glu-rich RE3, RE4, and RE5 peptides, and the vicilin-like protein are produced. *SP* represents a signal peptide.

arrival of the proproteins at the vacuoles. However, the possibility cannot be excluded that the propeptides exhibit some functions in the vacuoles after being removed from the precursor proteins, as the 4–6-kDa RE and C2 peptides are accumulated to act as functional proteins in the vacuoles.

VPE-mediated Cleavage at Asn-Gln Bonds of PV100 to Produce Multiple Seed Proteins with a Pyroglutamate at Their NH_2 Termini—Fig. 9 shows the hydrophobicity plot of PV100 and a hypothetical mechanism for vacuolar processing of PV100 to produce C2 peptide, RE peptides, and a vicilin-like protein. PV100 contains nine Asn-Gln bonds in the sequence. All six Asn-Gln bonds to be cleaved are located in the hydrophilic region of the PV100 sequence, whereas the other three non-cleavable Asn-Gln bonds are found in the hydrophobic region of the vicilin-like domain. The result is consistent with our previous data showing that VPE recognizes Asn residues that are located in the hydrophilic region and are exposed on the surface of precursor molecules (6).

We previously reported that one subunit of pumpkin 11S globulin has a pyroglutamate at the NH_2 terminus (41). NH_2-terminal sequencing of the proglobulin in the isolated PAC vesicles revealed that a cleavage of Asn-Gln bond by VPE produced a pyroglutamate at the NH_2 terminus of the 11S globulin subunits (11). VPE cleavage of an Asn-Gln bond gives a new NH_2-terminal Gln residue, which might be spontaneously converted into a pyroglutamate under the acidic condition in the vacuoles. Proteins with an NH_2 terminus blocked by pyroglutamate are resistant to aminopeptidases that are localized in the vacuoles. These results suggested that the Asn-Gln sequences not only provide sites that can be cleaved by VPE but also produce aminopeptidase-resistant functional proteins in the vacuoles.

In contrast to the accumulation of RE3, RE4, RE5, and C2 peptide in the vacuoles, none of RE1, RE2, RE6, or C1 peptides were detected in the vacuoles (Fig. 6). They might be sensitive to proteinases in the vacuoles. The C1 peptide has two Asn residues inside the sequence (Fig. 2) and can be attacked by VPE to be degraded. It should be noted that the RE3, RE4, RE5, and C2 peptides have no Asn residue inside their sequences, indicating that they are resistant to VPE. They are also resistant to aminopeptidase because of a pyroglutamate at their NH_2 termini. However, both RE1 and RE2, with a Glu residue at each NH_2 terminus, could be sensitive to aminopeptidases, if they were produced by cleaving an Asp^{118}-Glu^{119} bond and an Asn^{161}-Glu^{162} bond by VPE, respectively.

Further proteolysis for COOH-terminal trimming at the Asp residues must occur to make the final mature forms of the RE3, RE4, and RE5 peptides (Fig. 6). Recently, we have found that VPE also cleaves a peptide bond at carbonyl side of Asp, although the activity toward Asp is less than that toward Asn (data not shown). The finding is consistent with the report that the VPE homolog of vetch has a substrate specificity toward both Asn and Asp residues (42). Therefore, it seems likely that the COOH-terminal trimming of RE3, RE4, and RE5 peptides is also mediated by VPE.

Acknowledgments—We thank Prof. Shigekata Yoshida of Nagoya University Experimental Farm for growing the pumpkin plants. We are grateful to L. M. Melgarejo of the National University of Colombia for help with cDNA cloning, to Y. Koumoto and J. Morita for their skillful technique on column chromatography, to Y. Makino for helpful support on mass spectrometry and peptide sequencing, and to C. Nanba for growing pumpkin and castor plants.

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